PAF Regulate Blastocyst Development to Hatching Stage through PKC Activity in the Mouse

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ABSTRACT

The developmental regulation of the preimplantation mammalian embryos is a fundamental step for preparing the implantation and it may be regulated by several autocrine and paracrine factors including platelet-activating factor. PAF improved the embryonic survival and implantation but its role during blastocyst development is still largely unknown. In this study, the effects and the possible pathway of PAF on developmental regulation of blastocyst to hatching stage were investigated. Developmental pattern in hatching embryo was a concentration-response curve showing maximal activity at 1 nM PAF, with decreasing activity at higher concentrations. 50 μM 1-(5-isoquinolimnesulfonyl)-2-methylpiperazinme dihydrochloride (H-7), a PKC inhibitor, inhibited the progression of blastocyst to hatching embryo. In addition H-7 blocked the PAF effects on the blastocyst development. Besides tetradecanoylphorbol acetate (TPA), a PKC activator stimulated development of blastocyst to the hatching stage. These finding revealed that PAF support the blastocyst development to the hatching embryo. Also it is suggested that PAF action pathways in hatching supporting include the PKC signaling pathway.

(Key words : Blastocyst, Hatching, PAF, PKC)

INTRODUCTION

Many putative autocrine, paracrine and endocrine factors have been implicated in supporting preimplantation embryo development (Hardy and Spanos, 2002; O'Neill, 2005). Recent studies have implicated that platelet-activating factor (PAF, 1-O-alky-2-acetyl-sn-glyceryl-3-phosphocholine) (Lu *et al.*, 2004; Roudebush *et al.*, 2002), insulin-like growth factor I (IGF-I) (Markham and Kaye, 2003), IGF-II (Yaseen *et al.*, 2001), and epidermal growth factor / transforming growth factor α (Cai *et al.*, 2003) regulate the embryo development as autocrine factor. However their mechanisms of action and their temporal patterns of action are poorly understood during preimplantation period.

The early stage embryo synthesis the PAF with *de novo* synthetic manner and use it as autocrine and paracrine factors during early development (Stoddart *et al.*, 2001). PAF express in the embryos by a complete an autocrine trophic loop. PAF involve the embryonic cellular activity including embryonic metabolism (O'Neill, 2005), cell-cycle progression (Stoddart *et al.*, 2001) and embryo viability (Spinks *et al.*, 1990).

PAF activity regulation depends on deacetylation by phospholipase A2 (Tjoelker *et al.*, 1995; Prescott *et al.*, 2000), PAF-binding proteins (Ammit and O'Neill, 1997). PAF works through mainly its G-protein coupled membrane receptor (PAFr) (Ishii *et al.*, 2002). Embryo derived PAF induces transient increases in the intracellular calcium concentration within the 2-cell embryo. This action requires the presence of extracellular albumin and is inhibited by prior brief exposure of embryos to recombinant PAF acetylhydrolase or PAF receptor inhibitors (Emerson *et al.*, 2000).

Exposure of preimplantation stage mouse embryo results in higher implantation rates. Embryonic PAF is synthesized and released by rabbit and mouse embryos during the preimplantation period, with maximum levels at the expanded blastocyst stage (Angle *et al.*, 1988; Minhas *et al.*, 1993). Although the production of PAF by preimplantation embryos has been reported, physiologic roles of this potent mediator remain unclear.

Previous cavitation the role of PAF has been demonstrated but the effect of PAF on blastocyst development and its mechanism is not yet largely unknown. In this study, PAF was examined to determine the effect on blastocyst development to hatching embryo and it's receptor mediated signal molecules for the developmental progression.

MATERIALS AND METHODS

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Reagents

1-(5-isoquinolimnesulfonyl)-2-methylpiperazinme dihydrochloride (H7), a cyclic Ca^{2+} dependent protein kinase C (PKC) inhibitor (Takahashi *et al.*, 2005), and tetradecanoylphorbol acetate (TPA, porbol 12-myristate 13-acetate) were purchased from Sigma.

Experimental Animals and Embryo Collection

All animal procedures involved in this study were conducted according to NIH guidelines for the ethical use of animals in research. CD-1 mice were maintained on a 14-hr light and 10-hr dark cycle under standard vivarium conditions, and were supplied with food and water *ad libitum*. To induce superovulation, female mice (6~8 weeks old) were injected with 5 iu pregnant mares serum gonadotropin (PMSG, Sigma) followed after 48 hr by 5 iu human chorionic gonadotropin (hCG, Sigma). Compacted embryos were collected at 72 hr post hCG injection from oviduct-uterine junction by flushing with Biggers, Whitten and Whittingham medium (BWW; 94.6 mM NaCl, 4.78 mM KCl, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4 \cdot 7H_2O$, 1.71 mM calcium lactate, 21.58 mM sodium lactate, 0.3 mM sodium pyruvate, 25.07 mM $NaHCO_3$, 100 units/ml penicillin, 100 μg/ml streptomycin, pH 7.4, and 0.4% BSA).

Culture of Embryos and Treatment with Cytokine

Embryos (8~10 oocytes / drop) were cultured in 10 μl drops of BWW medium in mineral oil (Sigma) for 24 hr after collection (96 hr post hCG injection) at 37℃ in a humidified atmosphere containing 5% CO_2. To study the dose effects of platelet activating factor on hatching, blastocyst were cultured in BWW media containing PAF (1 nM, 500 nM, and 1 μM). The developmental progression was scored using a differential interference contrast microscope (Olympus, Japan).

Treatment of H7 and TPA

50 μM or 100 μM H-7 was treated on blastocyst (96 hr post hCG injection) and observed the embryonic stages under the microscope (Olympus IX70). On the other hand, to examine the blocking effect of H-7, H-7 was cotreated with 1 nM PAF on blastocyst. TPA was prepared as a 20 μg/ml stock solution in DMSO. Blastocyst stage embryos were treated with 20 ng/ml TPA for 48 hr and observed the developmental stages.

Statistical Analysis

In the maturation assay, each experiment was performed a minimum of 7 times. Data were analyzed by *t*-test or ANOVA and considered to be statistically significant at $P < 0.05$.

RESULTS

Table 1. PAF on the blastocyst development. Embryos were collected at 72 hr post hCG injection and exposed to various concentration PAF at 24 hr after collection

	Embryonic stages		
	Blastocyst (%)	Expanded (%)	Hatching (%)
Control	6/156(3.9)	21/156(13.8)	129/156(82.3)
1 nM	2/153(1.4)	8/153(5.6)*	143/153(93.0)
500 nM	1/119(1.0)	9/119(7.0)	104/119(88.4)
1,000 nM	8/122(6.6)	13/122(10.2)	102/122(80.1)

Embryonic stages were detected at 48 hr post treatment. * $P<0.05$.

Effects of PAF

To get a profile of exogenous PAF effects on the blastocyst development PAF was treated on blastocyst as mentioned at Materials and Methods. 1 nM PAF had most positive effect ($P>0.05$) on blastocyst development to hatching embryo (Table 1). The positive effects decreased by incensement of PAF concentration (Table 1).

H-7 Inhibit the Progression of Blastocyst to Hatching Embryo

To block the PKC activity in the blastocyst 50 μM H-7 was treated and got the result as shown Fig. 1 and Fig. 2. Some of the embryos developed to the hatching stage at 24 hr post H-7 treatment but significantly decreased the developmental rate compared with the control (51.3 % vs 77.2%) (Fig. 2). After then, the embryos stopped the deve-

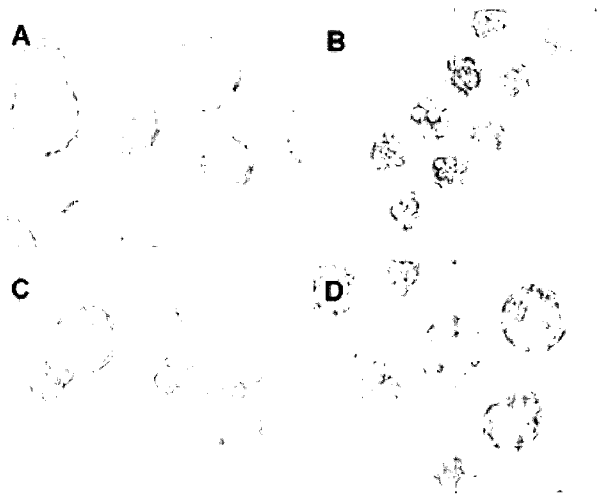

Fig. 1. Photomicrograph of the blastocyst after exposed to the PAF or H-7. PAF or H-7 was treated at 96 hr time point post hCG injection and cultured for 48 hr. A. Control embryos at 144 hr post hCG injection (same time with 48 hr after treatment). B. H-7 exposed blastocyst after 48 hr culture. Most of embryos were degenerated. C. PAF exposed blastocyst after 48 hr culture. D. Embryos after 48 hr culture which were cotreated with PAF and H-7. Many of the blastocysts stop developmental progression.

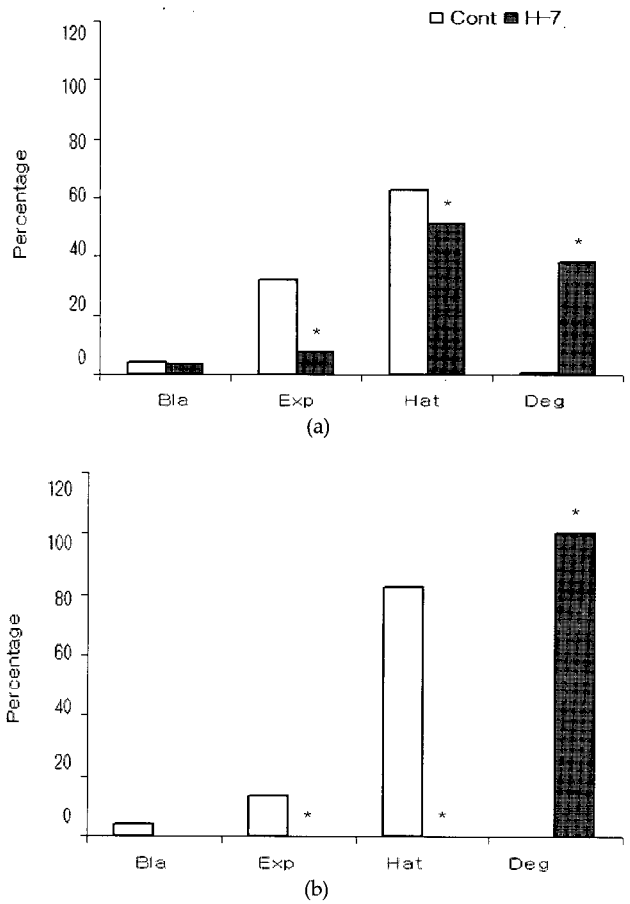

Fig. 2. Blastocyst development after treatment of H-7, PKC inhibitor (Number of blastocysts: 76). Embryos were collected at 72 hr post hCG and treated H-7 on only healthy embryos at 96 hr time point post hCG injection. Developmental stages were observed under the DIC inverted microscope at 24 hr (A) and 48 hr (B) after H-7 exposure. * $P<0.05$ versus control.

lopment and blastocoel was disappeared as shown in Fig. 1B. Blastocyst development was completely stopped by PKC inhibitor (Fig. 2).

H-7 Block the PAF Effects

PAF was cotreated with H-7 to know whether PAF to work through PKC. Interestingly H-7 severely blocked the PAF effects on blastocyst (Fig. 1D). Development of blastocyst to the hatching stage was significantly blocked by H-7 compared to control and PAF at 24 hr post PAF treatment (54.4% vs 77.2%) (Fig. 3). At 48 hr 52% of embryos were showed collapse of blastocoel and degeneration (Fig. 3).

TPA Stimulation of Blastocyst Development

A phorbole ester TPA is a well known PKC activator. Blastocysts were treated with TPA to activate PKC in blastocyst and to confirm the involvement of PKC on blastocyst development. As expected, the hatching rate

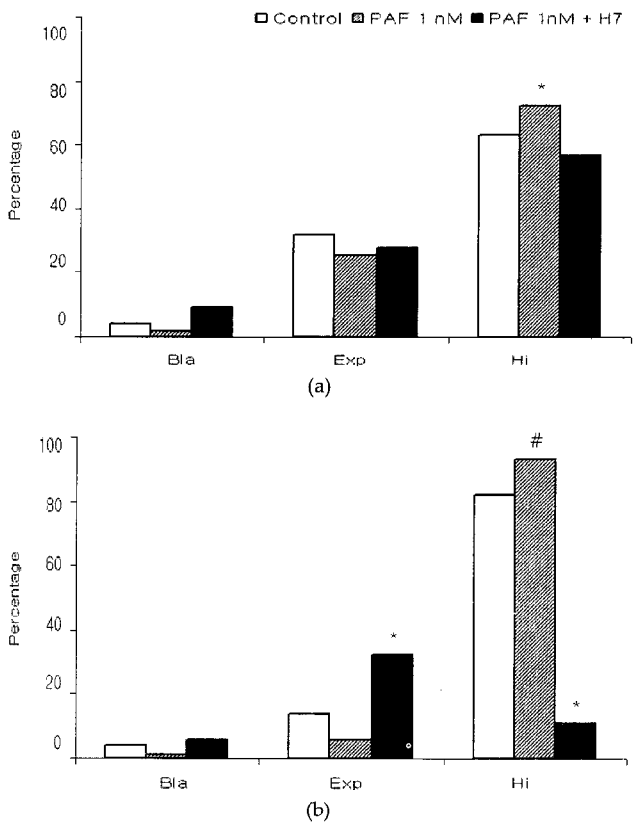

Fig. 3. Blastocyst development after cotreatment with PAF and H-7 (Number of blastocysts: 68). Embryos were collected at 72 hr post hCG and treated H-7 and/or PAF on only healthy embryos at 96 hr time point post hCG injection. Developmental stages were observed under the DIC inverted microscope at 24 hr and 48 hr after H-7 exposure. * $P<0.05$ versus control group, # $P<0.05$ versus both control group and cotreated group.

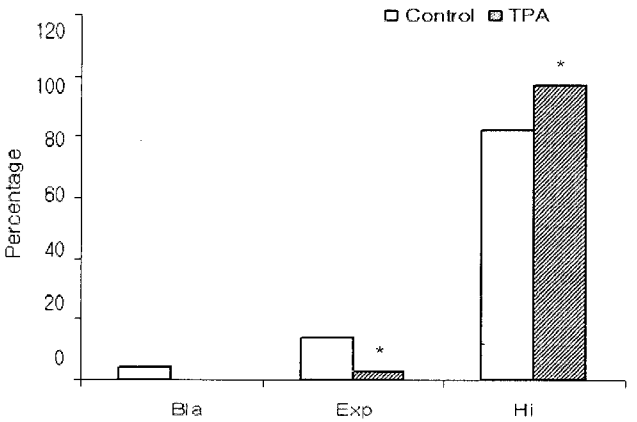

Fig. 4. Blastocyst development after treatemt of TPA, a PKC activator (Number of blastocysts: 72). Embryos were collected at 72 hr post hCG and treated TPA on only healthy embryos at 96 hr time point post hCG injection. Developmental stages were observed under the DIC inverted microscope at 48 hr after TPA treatment. * $P<0.05$ versus control.

was significantly increased in TPA treated group compare to the control (Fig. 4). The developmental rate to the

hatching stage was similar with 1 nM PAF treated group.

DISCUSSION

Early preimplantation embryos have high levels of PAF release (Roudebush *et al.*, 2002) and express PAF receptor (Stojanov and O'Neill, 1999). It has been suggested that the consequence for the embryo of PAF stimulation differs at different stages of development (O'Neill, 2005). Cell proliferation in mouse preimplantation embryos is stimulated by PAF with stage specific manner (Stoddart *et al.*, 2001). In addition to the previously known effects of PAF on the early preimplantation stage embryos, from this study, it is explored that the blastocyst stage embryo also response to PAF and 1~1,000 nM dose range of PAF can support the developmental progression to hatching stage. Also it is showed maximal encouragement of the blastocyst development to the hatching stage in 1 nM PAF.

The developmental progression rate of blastocyst to the hatching stage was stage specific and dose specific as seen in the PAF response profile on blastocysts. The PAF effects were giving various possibilities; even small increments above the optimal PAF concentration results in reduce embryo viability (Ryan *et al.*, 1990). The litter size of the PAF receptor over-expressed transgenic mice is reduced. On the other hand, PAF receptor knockout embryos cultured *in vitro* developed poorly compared to wild type embryos (Lu *et al.*, 2004). The modest reproductive phenotype of PAF receptor null mice and the apparent inverse agonism of PAF antagonists on embryos (O'Neil, 1995) were shown the transducing PAF's actions in the preimplantation embryo. Therefore dose-dependent blastocsyt developmental patterns showed that PAF characteristics exist from early preimplantation embryo to blastocyst.

The PAFr contains seven α-helical domains that span the plasma membrane and related with G-proteins. Depending on the cell types, multiple G proteins interact with the PAF receptor resulting in a myriad of distinct signaling pathways (Haribabu *et al.*, 1999; van Biesen *et al.*, 1996). PAF mediates a variety of physiological effects through coupled G-protein, including elevation of intracellular Ca^{2+} (Shukla, 1991), and induction of endothelial cell migration (Camussi *et al.*, 1995). Development of blastocyst to the expansion or hatching stage is regulated by various factors (Cheon, 2005). As seen the results, PKC inhibitor, H-7 inhibited the development of blastocyst to hatching stage and gave mortal effects in viability of the embryos. H-7 inhibited the PAF effects on the blastocyst development. Besides PKC activator stimulated the blastocyst development to the hatching stage. These result means that PKC is the downstream target molecule of PAF signaling pathway in the blastocyst.

PKC activity can be regulated by diacylgycerol, calcium, phospahatidylserine. Activated PKC phosphorylate target proteins that vary depending on the cell type. PKC involve in cavitation in preimplantation embryos via target proteins including Na^+/K^+ ATPase (Eckert *et al.*, 2004) and tight junction membrane assembly in the preimplantation mouse embryo (Eckert *et al.*, 2004). Several reports have shown that PKC may regulate actin cytoskeleton organization and dynamics (Cybulsky *et al.*, 2004; Keenan and Kelleher, 1998). Progression of blastocyst to the hatching stage was regulated factors like calcium (Cheon, 2005) and involved trophectodermal locomotion mediated actin filaments (Cheon *et al.*, 1999). Based on these reports it is suggested that PKC accelerated blastocyst development to the hatching stage through cavitation or actin filament mediated locomotion.

In summary, PAF induced responses, which reached a maximum at 1 nM PAF and declined at higher dose. PAF stimulate the hatching of the blastocyst, PAF acts through PKC, an intermediate protein in the signaling cascade that starts with PAF to ensure proper development. One of the suitable interpretations is that excessive PAF signaling is detrimental to the establishment of hatching or implantation.

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